



# Restricted genetic diversity in the ubiquitous cattle parasite, *Sarcocystis cruzi*

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## ABSTRACT

Although parasites of the genus *Sarcocystis* have likely cycled between bovine herbivores and canine carnivores for tens of millions of years, humans may have profoundly influenced the ecology and evolution of those prevalent in domesticated dogs and cattle. To preliminarily assess the possibility of such anthropogenic effects, we surveyed genetic variation in conserved (18S small subunit) and variable (ITS-1) portions of ribosomal DNA from a large sample of *Sarcocystis cruzi* occurring in taurine beef cattle raised in the United States and Uruguay, and compared these data to available homologues, including those reported from zebu cattle, water buffalo, and bison. For additional context, we compared the apparent diversity of cattle parasites to that reported from congeneric parasites in other hosts. We find that the *S. cruzi* of taurine cattle, whether derived from the Americas or Asia, are devoid of variability in the sequenced portion (80%) of the small subunit rDNA. By contrast, geographically limited samples of related parasites in other hosts, including those of wildlife, are more variable. At the adjacent ITS-1 locus, allelic distribution patterns did not indicate any regional barriers to gene flow, suggesting that the parasite may have been introduced to the Americas via a common source such as domesticated dogs or cattle. Thus, human impact on this parasite's distribution and diversification would seem to have been great.

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## 1. Introduction

By domesticating dogs and livestock, humans brought into continuous proximity animals whose wild ancestors had served as each other's predators and prey for millions of years. Dogs were among the first animals to be domesticated; skeletal changes differentiating them from their grey wolf forebears are evident in fossilized remains dating back 15,000 years (Lindblad-Toh et al., 2005; Vila et al., 1999). Approximately 9000 years ago, humans began domesticating livestock and dogs became widely used to herd and guard them (Clutton-Brock, 1999; Diamond, 2002).

Proximity to husbanded animals exposed humans to new zoonotic infections and sustained the transmission of highly communicable human pathogens (Wolfe et al., 2007). Although we are beginning to appreciate the impact that these pathogens had in shaping human colonial history (Diamond, 2002), it is less understood how humans have influenced the evolution of exclusively veterinary pathogens.

Here, we sought to determine how cattle and dog domestication may have influenced the biodiversity and biogeography of

parasites in the Apicomplexan genus *Sarcocystis*. Carnivores acquire *Sarcocystis* by eating infected prey. Prey become infected by grazing on vegetation contaminated with parasite cysts excreted by carnivores. We determined whether geographically disparate domestic cattle harbor especially homogeneous parasites by characterizing the diversity and geographic distribution of ribosomal DNA variants in *Sarcocystis* from North American, South American, and Asian bovines.

## 2. Methods

One hundred and twelve specimens of taurine beef were collected from various markets in the vicinity of Burlington, Vermont. Twelve specimens were imported from Uruguay, and the remaining 100 originated in the United States as Western ( $n = 85$ ) or Northeastern-raised ( $n = 15$ ) steer. By purchasing samples from different supermarkets on different days, we sought to minimize the possibility that any two specimens were derived from the same animal. Specimens were primarily sirloin cuts (98%). DNA was extracted from each 0.05–0.25 g beef specimen and from 11 negative controls (human liver or kidney) using Qiagen DN easy<sup>®</sup> tissue kit protocol for animal tissues with the following modification: penultimate elution in 200  $\mu$ l of buffer was followed by a final elution in half that volume, yielding a combined eluate of

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**Table 1**  
Primer sequences

Primer	Sequence	Position <sup>a</sup>
18S1F	GGATAACCGTGGTAATCTATG	138–159
18S7F	GGCTTGTGCGCTTGAATACTGC	774–795
18S10R	AGCATGACGTTTCTATCTCTA	1029–1051
18S11R	TCCTATGTCTGGACCTGGTGAG	1202–1223
SSU-EUK	TGATCCTTCTGCAGGTCACCTAC	1767–1785
18S14F	AGTGTCCGGTGAATTATTC	
ITS1-FR	ACATCCATTGCTG	

<sup>a</sup> With respect to the 18S rDNA of *Toxoplasma gondii* (EF472967).

approximately 300 µl. The final concentration of total extracted DNA was estimated using a Nano Drop ND-1000 spectrophotometer.

A 1626 bp portion of the ~1850 bp 18S rDNA (corresponding to bases 159–1785 of the *Toxoplasma gondii* homologue (EF472967)) was amplified from each specimen, as was the first internal transcribed spacer (ITS-1) of ribosomal nuclear DNA, using primers defined in Table 1. 18S rDNA was amplified using primer 2Lf (Yang et al., 2001a,b) in conjunction with either primer 1810R or 18S11R, and with primer 18S7F in conjunction with SSU-EUK modified from Primer B of Medlin et al. (1988) and the ITS-1 was amplified with 18S14F/ITS1-FR (Table 1).

Each 20 µl PCR reaction contained 0.25 units of Platinum High Fidelity Taq polymerase (Invitrogen), 1× PCR buffer, 0.6 mM MgSO<sub>4</sub>, 0.2 mM dNTP mixture, 0.5 µM each primer, and 2–2.5 µl template. After an initial 3 min denaturation at 94 °C, templates were subjected to 35 cycles of 94 °C for 30 s, 45 s annealing (53 °C for 18S rDNA and 43.2 °C for ITS-1 reactions), and a 75 s extension at 72 °C. A final 10 min extension at 72 °C completed each reaction. To ensure that amplifiable contaminants were absent during the extraction and amplification procedures, each round of PCR also included reactions containing DNA extracted from uninfected (human) tissues and reactions containing no DNA template. Positive controls run with each assay contained templates of *Besnoitia darlingi* and *Sarcocystis neurona*. Reaction products were electrophoresed in 1.4% agarose gels and visualized by ethidium bromide staining.

The amplified 18S rDNA was sequenced from 27 beef specimens infected with *Sarcocystis cruzi* derived from cattle in either the US ( $n = 24$ ) or Uruguay ( $n = 3$ ). After removing excess primers and dNTPs with ExoSap-IT<sup>®</sup> (USB Corp), PCR products were sequenced using amplification primers and Big Dye<sup>®</sup> Terminator version 3.0 or 3.1 on an ABI 3100 sequencer.

Sequence chromatograms were aligned and edited using Sequencer 4.2 (GeneCodes Corp., Ann Arbor, MI). Those rDNA homologues in GenBank<sup>®</sup> most closely resembling our *S. cruzi* sequence were identified using BLAST. A multiple sequence alignment of these was constructed using CLUSTALW as implemented in Vector NTI Advance 10. Minimum evolution trees were reconstructed from two matrices, one including 67 sequences at 492 ungapped positions, and another restricted to 59 nearly full-length sequences allowing comparison at 1208 ungapped positions. Genealogies were reconstructed under the criterion of minimum evolution from pair-wise Kimura 2-parameter distances using MEGA 3.1 (Kumar et al., 2004).

Variability of ITS-1 within *S. cruzi* isolates precluded attempts to directly sequence these ~870 bp products. Therefore, a cloning approach was adopted to characterize variants of this locus using the TOPO TA cloning kit (Invitrogen Corp., Carlsbad, CA). Plasmid DNA was purified from white colonies using the Qiagen QIAprep<sup>®</sup> Miniprep kit using the manufacturer's recommended protocol with the exception that samples stood for 5 min before final centrifugation. Plasmids were sequenced using Big Dye<sup>®</sup> terminator version 3.1 as described above.

### 3. Results

#### 3.1. 18S rDNA sequence analysis

The 18S rDNA sequences derived from cattle in the United States and Uruguay were almost completely lacking in genetic diversity. A single TA insertion occurred in one sequence (derived from specimen US 13) at position 725. (This and all locations refer to the complete 18S rDNA sequence from *T. gondii* (EF472967).) The only other suggestion of polymorphism came from 'double traces', confirmed by sequencing each DNA strand, indicating that both G and A occur at position 692 in at least seven of the specimens. Both of these positions reside in the E23 region of helices in which the greatest extent of variation has previously been described in other eukaryotic homologues (Van de Peer et al., 1998). The sequence characterizing 26 of our 27 new isolates exactly matches those previously reported from Chinese taurine cattle (GenBank accession nos. AF176933 and AF176934) and a Chinese water buffalo (AF176932) (Yang et al., 2001b) (Fig. 1).

Sequences of two other isolates, AF176935 (from a Chinese water buffalo) and AF017120 (most likely derived from a Swedish taurine cow-JT Ellis, pers. commun.), differed slightly from each other and from the sequence characterizing all American cattle isolates. The sequence of a parasite infecting a North American bison, SCRUI 18S1 (=AF006480) differed from all of these by an even greater extent (Fig. 1A). Thus, of five reported 18S rDNA haplotypes attributed to *S. cruzi*, only one is prevalent in the parasites of geographically disparate taurine cattle.

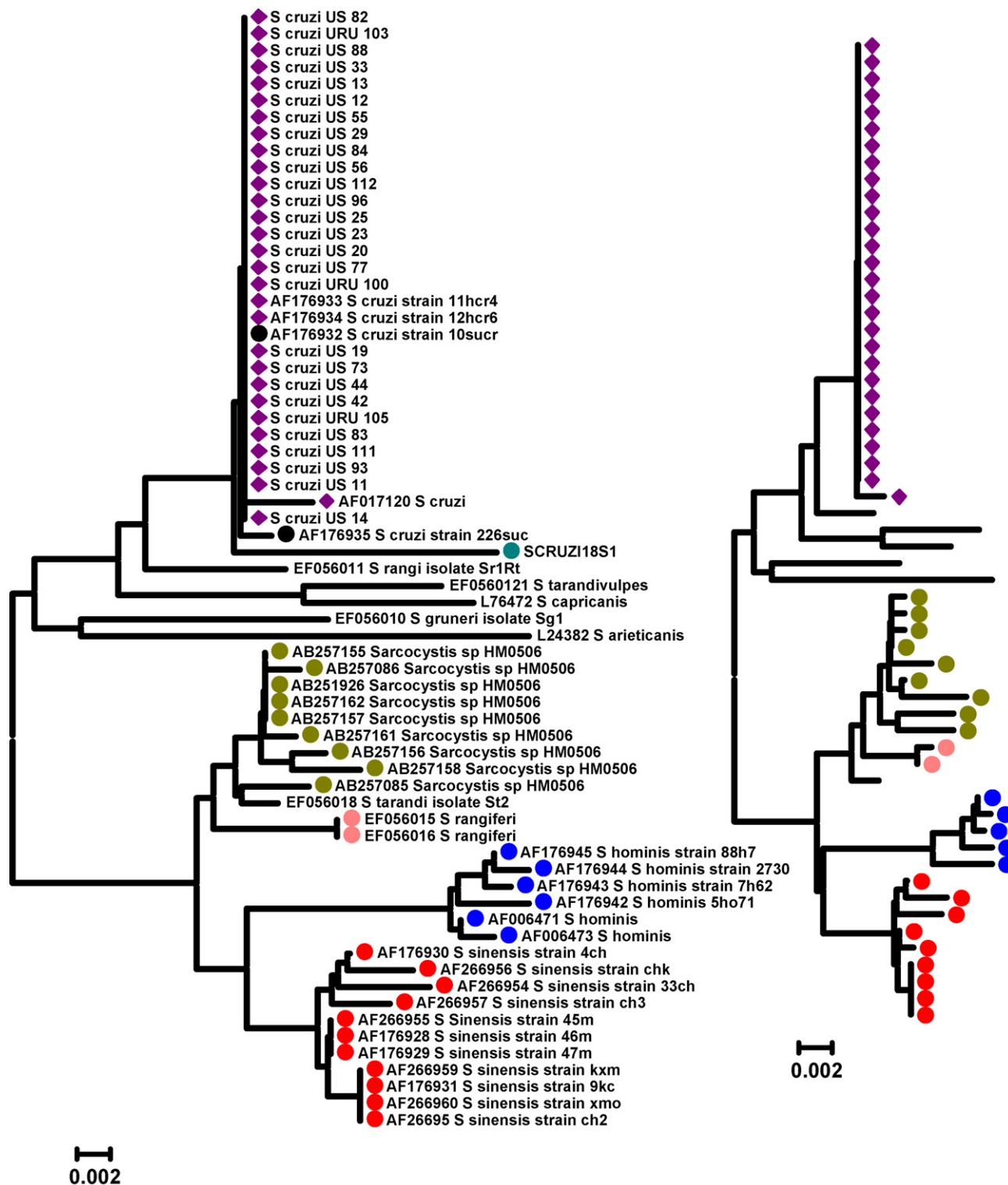
In comparison, modest genetic diversity characterizes isolates reported from other species of *Sarcocysts*, including *S. hominis*, *S. sinensis*, and an unnamed species of *Sarcocystis* occurring in *Cervus nippon yezoensis* in Hokkaido, Japan. Specimens identical over this 18S rDNA portion have occasionally been reported elsewhere. For example, certain haplotypes characterize groups of three *S. cruzi* specimens (Yang et al., 2001b). Similarly, one of four haplotypes of *S. hominis* has twice been deposited in GenBank<sup>®</sup>.

#### 3.2. ITS-1 rDNA sequence analysis

To characterize the more variable rDNA marker, the ITS-1, we sequenced one to five cloned copies of ITS-1 from each of twelve US or Uruguayan isolates (30 clones in total). These sequences resemble one another, but have no significant similarity to any sequences in GenBank<sup>®</sup> as judged by BLAST searches (excepting at their termini in the 18S and 5.8S rDNA subunits, which affirm derivation from a species of *Sarcocystis*). The ITS-1 encompassed variability within and among individuals (GenBank accession nos. EF622146–EF622176). Despite instances of coherence among an isolate's clones (for example, all five clones from US 15 defined a monophyletic group), other ITS-1 variants were distributed broadly among individuals and geographic regions (Fig. 2). Within some isolates (i.e. US 5 and US 24), ITS-1 diversity was nearly as great as that occurring in the entire sample; these clones were consequently distributed throughout the gene tree (Fig. 2). Finally, one clone from US 73 perfectly matched a clone from URU 100.

### 4. Discussion

Although domestication and dissemination of livestock might have exerted profound effects on the populations of veterinary parasites, this has seldom been tested empirically (Blouin et al., 1995; Donnelly et al., 2001; Morrison and Hoglund, 2005). Coccidian parasites are ubiquitous among vertebrates, and transmission among prey and predator hosts could conceivably have occurred for tens of millions of years. Old, large populations

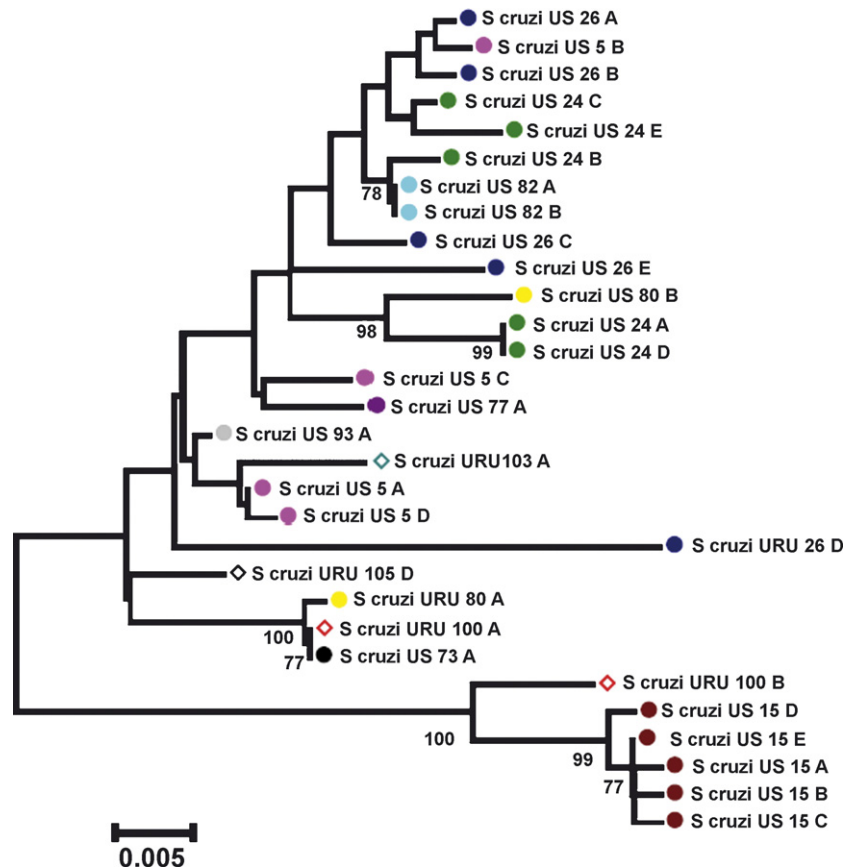


**Fig. 1.** Minimum Evolution tree reconstructed from Kimura 2-parameter distances of partial sequences of nuclear 18S rDNA. (A) Tree reconstructed from a matrix of 67 isolates at 492 ungapped bases. (B) Tree from which shorter sequences have been removed, resulting in a matrix of 59 isolates  $\times$  1208 ungapped bases. Symbols designate specimens of *S. cruzi* derived from domestic cattle (◆), water buffalo (●), or bison (●). Cattle isolates of *S. cruzi* appear less variable than isolates of *S. sinensis* (●), *S. hominis* (●), and or unnamed species from Sika deer (●).

would be expected to harbor substantial genetic variability, whereas populations that have experienced only recent growth might lack such variability. Domesticated livestock have undergone rapid population growth since the Neolithic period, and the same may be true of their parasites. Thus, these systems provide a

means to study the possible genetic consequences of animal domestication.

To estimate the genetic diversity among New World isolates of *S. cruzi*, we sequenced 18S rRNA from 27 new isolates of North American and Uruguayan beef, and compared them to published



**Fig. 2.** Minimum evolution tree reconstructed from Kimura 2-parameter distances of ITS-1 rDNA sequences of *S. cruzi* originating in either Uruguay (◇) or the United States (●). Each color denotes clones derived from a given specimen. Nodes supported by more than 75% of 500 bootstrap replicate analyses are indicated. In many cases, neither a clone's regional or individual identity predicts its location on this gene genealogy.

GenBank® sequences. As shown in Fig. 1, a uniform sequence (hereby designated the “cattle haplotype” of *S. cruzi*), characterized nearly all *S. cruzi* parasites from domesticated cattle. These cattle originated from diverse locations, including Uruguay, China, and two locations within North America. The preponderance of this haplotype in taurine cattle therefore appears geographically widespread. This haplotype has once been reported from a water buffalo (AF176932) (Yang et al., 2001a). Two other reported 18S rDNA sequences ascribed to *S. cruzi*, derived from a water buffalo and a bison, varied from this “cattle haplotype.” We recommend further sampling of globally dispersed *S. cruzi* isolates to verify the association of this haplotype with taurine cattle. Continued sampling in the Americas would seem unlikely to identify new sequence variants of 18S rDNA, since none were found in a sizable and geographically widespread sample.

The number of rDNA copies in each *S. cruzi* genome is unknown, although this parasite, like other apicomplexans, may employ slightly distinct ribosomes in its various life-history stages (Reddy et al., 1991). Here, we report for the first time the occurrence of two bases at a particular position of rDNA resulting from the direct sequencing of PCR-amplified genomic DNA extracted from infected beef. Because parasites were not individuated prior to sequencing, the possibility of mixed infections cannot be ruled out. Therefore, we cannot know whether intragenomic variation occurs among rDNA paralogues. Nonetheless, variation among paralogues or among alleles is vanishingly rare in *S. cruzi* 18S rDNA.

The haplotypes of *S. cruzi* from taurine cattle seems especially lacking in 18S rDNA variability when compared to comparably characterized isolates of *S. hominis*, *S. sinensis*, or an unnamed

parasite of Sika deer (Fig. 1). Their 18S rDNA sequences form moderately variable monophyletic clades. Because comparison sequences were derived by sequencing three independent PCR products (Fischer and Odening, 1998), we cannot simply attribute earlier reports of genetic variation to elevated rates of sequencing error. Nonetheless, more intensive sampling of other bovid and cervid hosts might reveal other widespread and uniform parasites. Only by prospectively sampling each host using equivalent methods would it be possible to definitively establish that the uniformity of *S. cruzi* in cattle is truly exceptional.

Finally, we sought to determine whether populations of *S. cruzi* in cattle have withstood prolonged barriers to gene flow since becoming established in the Americas by characterizing the geographic distribution of variants in a more variable rDNA marker, the ITS-1. We were interested in determining whether alleles of this locus exclusively occur in particular portions of their American range. Had each region been characterized by its own ITS-1 alleles, a sustained period of reproductive isolation would have seemed plausible. Instead, the overall variability of this locus hardly exceeds that present in individual samples of infected beef. Moreover, certain ITS-1 alleles occur in both the USA and Uruguay. Therefore, we found no evidence of regional barriers to gene flow since *S. cruzi* was established in the Americas.

Future studies, examining ITS-1 variation outside the Americas, could ascertain whether the Americas are demarcated by a distinct subset of polymorphisms.

The homogeneity of *S. cruzi* 18S rRNA in beef may reflect the domestication history of cattle. Extant cattle derive from particular progenitor populations which, despite periods of allopatric

isolation, have undergone substantial admixture. This may have provided opportunities for parasite dissemination through interbreeding and importation of cattle and/or dogs.

In summary, our analysis suggests that *S. cruzi* from domesticated taurine cattle conform to a specific lineage. That lineage's remarkable uniformity, and its exceptionally widespread geographic distribution, suggests that humans may have been responsible for its dissemination in cattle and dogs. To definitively understand the impact human beings have had on the evolutionary ecology of this veterinary parasite, more informative genetic markers will be needed. Nonetheless, these data provide the first evidence establishing the plausibility that, by domesticating dogs and cattle, human beings may have profoundly influenced the ecology and evolution of this veterinary parasite.

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